



Comparison of *interleukin 18* gene expression and its serum level between Iranian colorectal cancer (CRC) patients and healthy people

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Abstract

Colorectal cancer (CRC), also known as colon cancer, rectal cancer, or colon cancer is one of the important mortal cancers of the gastrointestinal tract in Iran and worldwide. Interleukin 18 is one of the cytokines that causes inflammation and plays a real role in the inhibition of CRC development and evaluation of its rate is significant in cancer patients. The purpose of the present study was to investigate the *IL-18* gene expression and its serum levels in Iranian CRC patients compared to healthy people. A total of 35 serum samples and 24 complete blood cells (CBCs) from CRC patients and as well as 20 control samples (healthy people) were collected from Al-Zahra hospital (Isfahan city, Iran) and serum and RNA specimens were isolated for molecular and serological investigations. The expression level of *IL-18* gene and its serum level for comparison in both CRC patients and healthy people groups were evaluated by quantitative real-time reverse transcription PCR (q-RT-PCR) technique and ELISA assay. The comparison of *IL-18* gene expression and its serum level in blood of the CRC patients and healthy people using real-time PCR and ELISA techniques were showed that this cytokine level was increased significantly in cancer patients ($p < 0.05$). According to the significant role of IL-18 as a pro-inflammatory cytokine in developing inflammation of gastrointestinal tract and cancer and increasing of this cytokine expression in the results of our research in CRC patients compared to the control subjects, for diagnosing or pre-screen of CRC and other cancer patients, it would be better IL-18 expression level is check in laboratories.

Keywords Colorectal cancer · Inflammation · Interleukin 18 · Real-time RT-PCR · ELISA

Abbreviations

CRC	Colorectal cancer
CBSc	Complete blood cells
q-RT-PCR	Quantitative real-time reverse transcription PCR

Introduction

Colorectal cancer (CRC) engages the colon or the rectum and it is the third most common cause of cancer death in different countries especially in Iran and after lung cancer, it is also the second cause of cancer deaths in the industrial countries

(Rafiemaneh et al. 2016; Siegel et al. 2017). It is estimated that about one million new cases of CRC are detected worldwide every year and nearly half a million of them die due to the disease. Australia, New Zealand, North America, Canada, and parts of Europe have the highest rate of CRC and Africa, Central and South Asia, China, India, and South America are low incidence areas (Center et al. 2009; Jemal et al. 2011; Schreuders et al. 2015). After lung and prostate cancers, the prevalence of CRC in Iranian men is in the third place (about 5%) and its prevalence after breast cancer in women is 5.5% (Jemal et al. 2011; Nejadtoghi et al. 2017). The incidence of CRC in middle-aged people (over fifty years) and especially in men is high and in recent years the incidence of this cancer in people under fifty years it is common (Dolatkhah et al. 2015; Keyghobadi et al. 2015; Abdifard et al. 2016).

CRC has two forms including hereditary (familial) and sporadic which approximately 80% are sporadic and 20% are hereditary (Center et al. 2009; Montazer Haghighi et al. 2009; Nejadtoghi et al. 2017). Risk factors for CRC are including obesity, low intake of vegetables and fruits, inactivity and cigarette smoking (Center et al. 2009). Biological

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treatments are one of the different therapies which can be used for treating CRC. Human nutrition plays an important role in the development of CRC and consumption of foods with plenty of fiber, antioxidants, and omega-3 fatty acids can be helpful to prevent or improve the conditions of this cancer (Baena and Salinas 2015).

Inflammation is one of the important factors that play an important role in the production of cytokines against expansion of colorectal cancer and can also improve the condition of the patient and decrease of tumor progression by production of interferon from T-cells and increase of interleukins 7, 18, and 17 (Kostic et al. 2013; Nemati et al. 2015; Brennan and Garrett 2016; Krzystek-Korpacka et al. 2017).

Interleukin 18 (IL-18) is a pro-inflammatory cytokine and important regulator of the inherent and acquired immune responses (Ramazi et al. 2014; Nielsen et al. 2016). This cytokine can play an antitumoral and tumor role in CRC by stimulation of positive T cells to the production of interferon gamma (IF- γ). IL-18 activates neutrophil, produces free radicals of oxygen, and releases cytokines (Lippitz 2013; Yadav et al. 2015). IL-18 not only induces T helper cells cytokines, but also stimulates the T cell cytokines production related to T helper cells type I, including IL-10, IL-13, and IL-4, and along with IL-13 and IL-4 plays an important role in inflammatory and immune processes. *IL-18* gene is located on chromosome 11 q22 and this gene is associated with a high level of immunoglobulin E (Gracie et al. 2003; Yadav et al. 2015; Southcombe et al. 2015). Although IL-18 structurally related to the IL-1, unlike IL-1 its main biological function is increased by IF- γ production, T cells, and the differentiation induction of IF- γ . Macrophages and dendritic cells are the main sources of IL-18 (Yang et al. 2015; Hammer et al. 2018). According to the role of IL-18 as an anti-tumorigenic cytokine, this study was aimed to comprise the *IL-18* gene expression and its serum level in CRC patients and healthy people.

Materials and methods

Sampling and ethical approval

In this case-control study, the ethical approval was obtained on September 20th, 2016 from the Ethical Committee and Research Deputy of Islamic Azad University of Shahrekord Branch, Iran. Then, the written consents it was gathered within three months and the questionnaires were filled out. In the testimonial forms, the details of each patient included age, sex, smoking, history of the disease, and stage of the disease were taken from the case file. A total of 35 serum samples and 24 CBCs from CRC patients (19 men with mean age of 61.78 years and 16 female with mean age of 53.37 years) and as well as 20 control samples (healthy people) were

collected from Al-Zahra hospital (Isfahan city, Iran). In CRC patients, 20 people had a story of the digestive disease and 15 people without a history of the disease. A 5 mL of peripheral blood samples from each case and control people were taken and transferred into two CBC and normal tubes. After centrifugation for 20 min at 3000 g, the serum was isolated immediately and the remaining complete blood samples were used for RNA extraction. All collected samples were evaluated by Real-Time PCR and enzyme-linked immunosorbent assay (ELISA) techniques to determine the *IL-18* gene expression and serum level of this interleukin.

RNA isolation and cDNA synthesis

Total RNA was isolated from peripheral blood specimen of both patients and control people using Jena Bioscience RNA extraction kit (Jena Bioscience, Jena, Germany) according to the manufacturer's protocol. The quality and quantity of each extracted RNA sample were measured by Thermo Scientific™ NanoDrop 2000 (Wilmington, DE, USA) at a wavelength of 260/280 nm and 1% agarose gel electrophoresis according to the method described by Sambrook and Russell 2001. Then, each RNA sample (0.2 μ g) was reverse transcript to cDNA by Yekta Tajhiz Azma cDNA synthesize kit (Yekta Tajhiz Azma, Tehran, Iran) using Oligo (dT) 18 primer according to the company's recommendations. The cDNA synthesize mixture was heated to 85 °C for 5 s and then was incubated at 42 °C for 60 min, followed by heat inactivation for 5 min at 85 °C. Each cDNA sample was subjected to reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative RT-PCR (q-RT-PCR) analysis.

Conventional reverse transcriptase PCR (RT-PCR)

The specific oligonucleotide primers for amplification of *IL-18* and *GAPDH* (as a reference) genes were designed using Gene Runner software version 3.05 (Hastings Software Inc. Hastings, NY, USA) and the specificity of primer sequences was verified using the basic local alignment search tool (BLAST) of the National Center for Biotechnology Information (NCBI) GenBank online database and finally were ordered to Macrogen, Inc. (Seoul, South Korea) (Table 1). Amplification was performed using FlexCycler² PCR Thermal Cycler (Core Life Sciences, Germany). In brief, RT-PCR reaction was performed in a 25 μ L final volume containing dNTPs at 200 μ M each, 2.5 mM of MgCl₂, 0.05 unit/ μ L of *Taq* DNA polymerase (all Cinna Gene, Iran) and 1 μ g of template cDNA and diffusion water. In this step, a negative control was contained all reagents without cDNA that replaced by equal volume of diffusion water. The PCR conditions consisted of initial denaturing at 95 °C for 5 min, 35 cycles of 94 °C for 30 s (initial denaturation), 63 and 65 °C at 30 s for GAPDH and IL-18 primers annealing, respectively

Table 1 The sequence of designed primers used for the q-RT-PCR reaction

Gene	Primers name	Sequence	Annealing temperature (°C)	Product length (bp)	GenBank accession number
<i>IL-18</i>	IL-18-F	5'-GACGCATGCCCTCAATCC-3'	65	105	XM_011542806
	IL-18-R	5'-CTAGAGCGAATGGTGCAATC-3'			
<i>GAPDH</i>	GAPDH-F	5'-GGAAGGTGAAGGTCGGAGTC-3'	63	185	NM_001289746
	GAPDH-R	5'-TCAGCCTTGACGGTGCCATG-3'			

and 72 °C for 30 s (extension), followed by a final extension at 72 °C for 5 min.

The electrode buffer used was TBE (10.8 g of Tris-base 89 mM, 5.5 g of boric acid 2 mM, EDTA (pH = 8.0) 4 mL of 0.5 M EDTA (pH = 8.0) that were combined in enough H₂O and were stirred to dissolve) and 10 µL aliquots of RT-PCR products were loaded on the 1% agarose gel electrophoresis and a constant voltage of 80 V for 30 min was used for product separation. Thermo Scientific GeneRuler 100 bp DNA Ladder (San Jose, California, USA) was used as a DNA molecular weight to determine the amplified cDNA products for confirmation of cDNA synthesis. After ethidium bromide staining the agarose gel was exposed to UV light and the picture was taken by UVIDoc gel documentation system (Uvitec, UK).

Q-real-time-PCR analysis

The expression level of *IL-18* gene in CRC patients and healthy people compared to the *GAPDH* gene (housekeeping gene as a control) were measured by q-RT-PCR. The q-RT-PCR reaction mixture was done in a final volume of 25 µL containing 12.5 µL of 1 × SYBR-green master mix (Applied Biosystems), 100 ng of diluted cDNA (1.5 µL), 2 µM of each appropriate primer (1 µL), and 9 µL of infusion water. Real-time PCR quantification was performed using 45 cycles on the Corbet Rotor-Gene 6000 (Corbett, Australia). The thermal cycling program was as follows: an initial denaturation at 95 °C for 2 min followed by 45 cycles of denaturation at 95 °C for 10 s, primer annealing at 61 °C for 15 s, and extension at 72 °C for 20 s. The melting temperature analysis was also performed from 55 °C to 95 °C at a transition rate of 0.1 °C/s at the end of an amplification reaction. The relative expression change of the target gene (*IL-18*) compare to the control gene (*GAPDH*) was revealed by the cycle of threshold (Ct) values using standard curve analysis and $2^{-\Delta\Delta C_t}$ relative quantitative method.

ELISA test for evaluation of IL-18 serum level

IL-18 serum levels in 40 µL of serum of CRC patients (35 samples) and healthy people serum (20 specimens) were determined by IL-18 ELISA kit (Eastbiopharm Co., Germany)

according to the manufacturer's guidelines. Standardization was performed using a standard solvent and dilution buffer at a concentration of 4, 8, 16, and 32 ng/L. The procedure was Sandwich ELISA assay and IL-18 concentration in serum samples of all wells of the plate was measured at 450 nm wavelength by Stat Fax-2100 ELISA plate reader (Awareness Technology, Palm City, FL).

Statistical analysis

Each experiment was repeated at least three times. The statistical significance of the relative expression changes of *IL-18* and *GAPDH* genes, and as well as IL-18 serum levels in CRC patients compared to the healthy people were analyzed by t-test analysis and least significant difference (LSD) multiple comparison tests in Social Sciences software (SPSS, Inc., Chicago, IL, USA) version 22 followed by independent T-test to examine the relationship and the significance of the data. Also, all graphs were drawn with the GraphPad Prism software (GraphPad Software, version 5.01, San Diego, CA, USA). All data were considered at a significant level of p value < 0.05.

Results

Patient's information

The details on the age structure, sex composition, and history of the disease are shown in diagrams (Fig. 1).

Conventional RT-PCR assay for verification of cDNA synthesis

The RT-PCR reaction was performed for *GAPDH* (reference gene) and the *IL-18* gene amplification to ensure the cDNA synthesis was verified for all specimens. The amplified cDNAs of both genes were investigated on 1% agarose gel electrophoresis and fragments with a length of 105 and 185 bp were revealed for *IL-18* and *GAPDH* genes, respectively and confirmed the precision of the synthesized cDNA (Fig. 2).

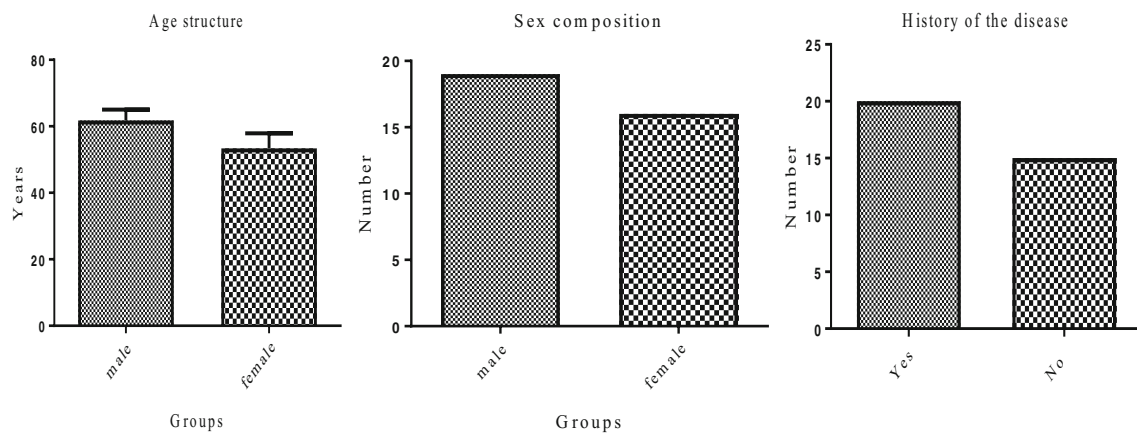


Fig. 1 Information about the age structure, sex composition, and history of the disease in CRC patients

Gene expression analysis

The analysis of *IL-18* gene expression compared to *GAPDH* gene in CRC patients and the healthy people using q-real-time RT-PCR using T-test analysis were evaluated. The results were showed that the expression levels of the *IL-18* gene in CRC patient was increased statistically significant compared to the healthy people using t-test analysis (p value <0.05) (Fig. 3).

ELISA test results

The comparison of IL-18 serum level by ELISA assay was indicated that the level of this cytokine in the serum sample of CRC patients was significantly increased compared to its serum level in healthy people by T-test analysis (p value <0.05) (Fig. 4).

Discussion

CRC after breast, prostate, and lung cancers is one of the most common cancers in developing countries and related to people

nutritional status and genetically factors (Kamangar et al. 2006). Also, the high incidence of CRC in the Iranian population has been reported (Dolatkhah et al. 2015). The statistics in Iran indicate that half of the patients are under age 50 years and the incidences of CRC in Iran are increasing (Malekzadeh et al. 2009; Safaei et al. 2012; Delavari et al. 2014). An inflammation of the intestine plays a very important role in the development and progression of CRC. Important agents such as age over 50 years, alcohol consumption, history of rectum, breast and ovarian cancers, polyps, hereditary syndromes and inflammatory bowel, digestive diseases like familial adenomatous polyposis and non-polypoidal adenomatous colon (Lynch syndrome) are risk factors for CRC (Cavestro et al. 2018; Mohapatra et al. 2018). IL-18 is one of the important cytokines as it works as an antitumoral role in CRC patients that it interferes with the IL-12 synergistic effect and causes to inducing of IF- γ production and thus leads the immune response to type I of T cells (Eaton et al. 2003; Mager et al. 2016; Mojic et al. 2018). Thus, in the present work, the gene expression and serum level of IL-18 in Iranian CRC patients compared to the healthy people were evaluated. After the collection of peripheral blood samples from each CRC patients and control people from one hospital in Isfahan city (Iran),

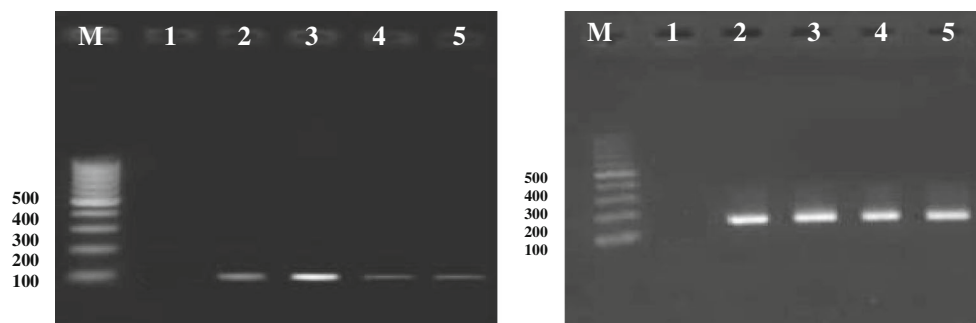


Fig. 2 The cDNA synthesis authenticity of *IL-18* (left) and *GAPDH* (right) in CRC patients and healthy people by RT-PCR technique on a 1% agarose gel electrophoresis (in both gels the lane M: 100-bp

molecular weight marker (Roche, Mannheim, Germany), lanes 1: negative controls (without template cDNA), and lanes 2 to 5: amplified cDNA for *IL-18* (105 bp) and *GAPDH* (185 bp), respectively)

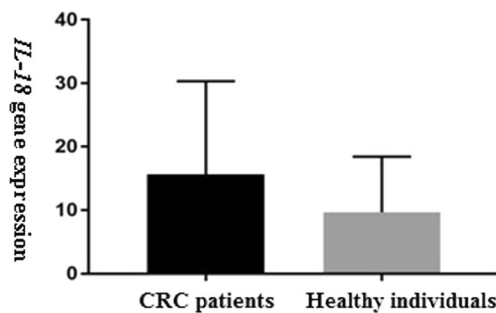


Fig. 3 The compression of *IL-18* gene expression in CRC patients compared to the healthy people using t-test analysis

these specimens were used for molecular and serological investigations. The findings were showed that the *IL-18* gene expression level in CRC patient was increased statistically significant compared to the healthy people ($p < 0.05$). In addition, the ELISA assay was indicated IL-18 cytokine level in serum samples of CRC patients was more than its serum level in healthy people.

In many studied the effects of IL-18 in cancer patients has been studied. In the study of Bagheri et al. 2014 the expression level of *IL-6* cytokine mRNA in 51 biopsy samples of 18 patients with stomach inflammation in Chaharmahal Va Bakhtiari province (southwestern Iran) was evaluated using real-time PCR. They indicated that IL-18 may play an important role in inflammatory response and progression of Th1 response to *Helicobacter* infection and production of clinical outcomes. Also in the present study, increasing of genomic and serum levels of IL-18 and its effects on Th1 pathway and production of inflammatory cytokines in CRC patients were observed and according to these findings IL-18 has a significant role in cancer patients and bacterial infections. Medina et al. 2014 indicated a possible role of IL-18, IL-18BP, and IL-18R in the pathogenesis of epithelial ovarian carcinoma. In different studies by Ghorbani and coworkers in Iranian people and Zhao and colleagues in Chinese population the association between polymorphism of interleukin 17 (IL-17F) and IL-17A and increased susceptibility to gastric cancer

evaluated and their researches demonstrated that these interleukins are genetic risk factors in the predisposition to gastric cancer (Ghorbani et al. 2012; Zhao et al. 2016). In our study, only the expression level of *IL-18* and its serum range in CRC patients compared to the healthy people in Isfahan city (Iran) were examined and the relation between increases of this cytokine in cancer patients was observed. In another study, the high expression level of IL-8 in CRC cells using real-time-PCR technique was reported. IL-8 not only provides the proliferative benefits but also potentiates the metastasis of intestinal cancer cells. IL-8 cells expressing significantly increase tumors of the control cells by increasing capillary density. In general, these findings indicated that IL-8 over-expression reinforces tumor growth, metastasis, chemical resistance, and angiogenesis (Ning et al. 2011). While, in the present study, the IL-18 expression level in the people with intestinal tumors was increased significantly. Nakanishi and colleagues examined the effect of IL-18 and Th1 cells on inflammation. The results of their study indicated that Th1 cells expressing IL-18, a response to antigen and IL-2 induce IFN- γ , and cause the further increasing of IFN- γ production after IL-18 stimulation. Their findings illative the important role of IL-18 and its synergism effects on IFN- γ production in cancer patients like CRC (Nakanishi et al. 2010). In another study, it was found that the serum IL-18 and vascular endothelial growth factor (VEGF) levels were significantly higher in patients with prostate cancer before operation with respect to healthy controls ($p < 0.05$) and these factors may be a useful prognostic marker in patients with prostate cancer (Nong et al. 2010). While the findings of our study showed that IL-18 expression and serum levels in CRC patients compared with normal group increased significantly ($p < 0.05$) and can be used probably in diagnostic studies of cancer. In the recent study, IL-18 expression significantly and positively correlated with breast cancer patients and it was suggested that IL-18 can be used as a potential predictor marker and guidance physicians for recommendations to newer treatment (Parikh et al. 2017). While, in the present work in Iranian CRC patients (Isfahan city), these findings were observed.

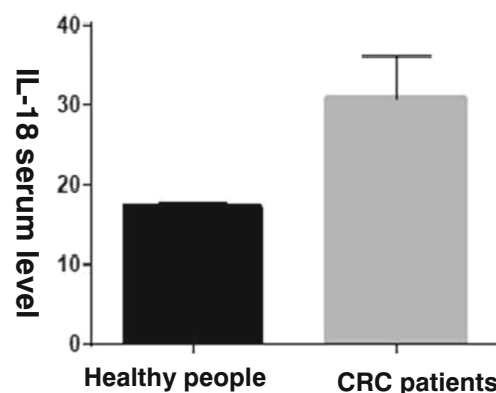


Fig. 4 The comparison of IL-18 serum level in CRC patients and healthy people by ELISA assay using T-test analysis

Conclusions

Due to the findings of this study, the increase of IL-18 in the blood of CRC patients compared to the healthy group was observed. Also, according to the IL-18 association with inducing of IFN- γ production and the progression of the cancers the enhancement of IL-18 expression in cancer patients it can be used as a biomarker for cancer diagnosis in the therapeutic strategy against cancers. Therefore the evaluation of serum and genomic expression of this cytokine for clinical tests in cancer patients is necessary.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest in this study.

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